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IMMUNIZATION BY MEANS OF CULTURES OF TRYPANOSOMA LEWISI.* †

F. G. Novy, W. A. Perkins, and R. Chambers.

(From the Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.)

In a previous paper[1] it was shown that cultures of *Tr. lewisi* when plasmolyzed in collodium sacs, against distilled water, underwent changes resulting apparently in complete solution or rather disintegration of the organisms. Three or more injections, on alternate days, of such plasmolyzed cultures protected young rats against a minimal infective dose of virulent blood. Immunity could therefore be produced by the injection of disintegrated cultural trypanosomes.

At the time it was also pointed out that cultures of *Tr. lewisi* and *Tr. brucei*, even after they had passed through a hundred generations or subcultures, in the course of two years, do not become attenuated by such prolonged consecutive passage but readily infect. This statement is only partially true, since the organisms on prolonged cultivation actually do suffer some loss of virulence or capacity to infect. A culture of *Tr. brucei* in the 100th generation will infect rats but the period of incubation is somewhat lengthened, and while death may occur within two or three weeks, it may not result until several months have elapsed from the time of inoculation; the longest survival is one which is at present in its 253d day. Obviously, the conditions which obtain in cultivation, notably the composition and reaction of the medium, the temperature and duration or age of culture, are not without appreciable effect on these organisms.

From the beginning, when the cultivation of *Tr. lewisi* and *Tr. brucei* was first effected, we have looked forward to the production of attenuated strains which could be utilized in preventive inoculations. It seemed reasonable to believe that the cultures of

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[1] *Proc. Soc. Exp. Biol. and Med.*, 1907, 4, p. 42.

pathogenic protozoa, like those of bacteria, would be found available to some extent in the production of immunity. The fact that spontaneous recovery may occur in protozoal diseases, as is the case with relatively insusceptible species, indicates the establishment of an acquired immunity, and hence the inherent capacity possessed by such an animal to protect itself against the given parasite. The trypanosomal infection of the common rat is a most excellent illustration of this fact, since it only exceptionally proves fatal. In the course of some weeks, or months, the rat gets rid of the trypanosome and becomes immune to a subsequent inoculation. It is evident, therefore, that the cultures of this parasite are particularly suitable for work bearing upon trypanosomal attenuation and immunity. With this organism it has been possible to demonstrate two important facts, first, that attenuation can be secured, and second, that such living, attenuated cultures confer a solid and permanent immunity.

The culture of *Tr. lewisi* which we have used was isolated from a rat September 5, 1905, since which time it has been maintained on ordinary blood agar medium, at 25°. It is at present in the 313th generation. At first, the culture was readily infective; the growth from a single tube being sufficient to produce typical infection in the rat. This infective property persisted for a considerable period and was frequently utilized to supply infected rats for the laboratory. After a long interval, in March, 1909, this procedure to secure a rat infection was again resorted to, the culture then being in the 158th generation. Contrary to expectation, no infection resulted. During the next few weeks no less than 17 rats were tested by intraperitoneal injections, each receiving from two to four cultures. In every case, as before, the result was negative, although daily examinations were made for an entire month. These tests clearly demonstrated that this stock culture of *Tr. lewisi* had completely lost its capacity to develop within the rat. In other words, it had become fully attenuated.

The many inoculations made with this culture during the past three years have invariably failed to produce infection. The result is the same whether the contents of a single tube or of 50 tubes is injected into the rat. Such cultures, grown at 25° for seven to 10 days, were extremely rich in flagellates which at times formed a thick slimy covering on the blood agar medium. Hence, the quantity of flagellates injected was at times enormous, and yet, notwithstanding this fact, no infection could be produced. The microscopic appearance of the cultures was in no wise different from that of the early generations which gave positive results, but the parasitic property of the organism was definitely lost.

All attempts to restore the infectious character to the culture have been fruitless. Even when the culture was placed in collodium sacs, in the peritoneal cavity of rats, it failed to multiply. Possibly further efforts in this direction may be successful.

While it is true, as has been emphasized above, that the cultures are no longer infective, it is an interesting fact that the majority of the inoculated rats do show in their blood, within the next day or two, a small number of fairly typical blood trypanosomes. These, however, do not increase in numbers and usually disappear within 48 hours after the injection. After that they are not to be found even though most careful examinations are made for a month or more. With the hope of restoring the virulence, the blood of rats showing these early trypanosomes was drawn from the heart and injected[1] into clean young rats and was also planted at the same time on blood agar. Much to our surprise, it was found that such blood failed to infect the rats and also failed to give cultures in the tubes.

The failure to secure a growth on the blood agar medium was particularly surprising, since, as is well known, this cultural method is capable of revealing trypanosomes even when prolonged microscopic examinations are negative. In all, about 12 rats which showed these transitory trypanosomes were cultured without obtaining the slightest indication of a growth. Similarly, the inoculation of nine rats with such blood, the doses varying from 0.25 c.c. to 3 c.c., failed to infect. Here, as above, the negative result could not be ascribed to the small number of trypanosomes, for it can be readily shown that the normal trypanosomal blood will infect, when diluted, to such an extent that the parasites can scarcely be detected under the microscope. Likewise, the susceptibility of our rats could not be called into question. In all of our work special care was taken to use only young rats, weighing from 60 to 90 gms., which had been repeatedly examined for two or more weeks and thus found to be free from natural infection. When used as controls, the injection of 1 c.c. of a 1:10,000 dilution of blood containing *Tr. lewisi* invariably gave a rich infection.

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[1] All injections unless otherwise indicated were given intraperitoneally.

It is evident, therefore, that the trypanosomes which appear transitorily in the blood of rats after the injection of a culture are incapable of further multiplication or reproduction. They are sterile or abortive forms, and without doubt their further study will prove of interest. It would seem as if some of the cultural trypanosomes still possessed a tendency to develop into the blood form, but the resulting individual through lack of inherent vigor or failure of fertilization (?) is unable to develop further and soon disappears.

It is probable that these abortive forms are present in the blood of all rats inoculated with the cultures. They are, however, easily overlooked and for that reason they have been found in only 75 per cent of the rats examined. They have been observed in the blood on one occasion five hours after the injection; at other times within 12 hours, but more often between 24 and 48 hours. Only exceptionally have they been seen as late as 72 hours and once they were observed to be present for four days. As already stated, the abortive forms are to be found in small numbers only. At times, after a prolonged search, but a single one can be found; more often, one can be found for every five or ten fields (No. 7 objective); and, rarely, one for every two fields examined. The number of abortive forms is independent of the dose injected, for in rats which received 50 cultures they were no more numerous than when a single culture was given. Likewise, the age of the culture seems to have no influence upon the number of these forms.

IMMUNITY.

As already stated, our previous studies have shown that cultures of *Tr. lewisi* when plasmolyzed[1] were capable of producing immunity in rats. It was therefore to be expected that the living attenuated cultures would behave in a similar way and confer protection against the blood trypanosome. The experimental work in this direction demonstrated that such was the case and that an active immunity was developed in animals which had received single or multiple injections of the living cultures.

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[1] This method of preparing a vaccine, though not generally applicable, has been utilized in this laboratory by Dr. J. G. Cumming, in charge of the University Pasteur Institute, in the preparation of a safe antirabic vaccine. His experiments have shown that the dialyzed virus protects animals even more efficiently than does the desiccated virus prepared in the ordinary way.

In order to obtain absolutely conclusive results it was necessary to employ young rats, preferably those raised in the laboratory, which repeated examinations for at least two weeks before use had shown to be free from the natural infection. In the beginning, when rats which had been purchased on the market were used, it not infrequently happened that the control animals in an experiment would fail to become infected, thus invalidating the immunity test. This difficulty was entirely overcome by employing only carefully tested young rats.

Another point of importance, not infrequently lost sight of in immunity tests, is the use of a very small but surely infective dose of the virulent organism, for the reason that a given degree of immunity may be effective against a minimal infecting dose but not against a hundred or thousand doses. A very large dose of virus may readily break down a slight immunity and thus lead to the erroneous conclusion that no protection exists. With this consideration in mind, we have as a rule employed the test virus in a dilution (in 0.85 per cent salt solution) of 1:10,000. One cubic centimeter of this suspension has never failed to produce a rich infection in clean young rats. In some instances, as shown in the tables, suspensions of 1:200 or 1:2,000 were used.

On examination of Table 1, several interesting facts will be noted. When the interval between the injection of the vaccine and the test dose is but two or three days (rats Nos. 3, 20, 21, 9, 27) a rich infection results, showing that little or no immunity exists at that time. Possibly a smaller dose of the test virus might have shown some immunity at this early date, because this is certainly evident on the fourth day (Nos. 5 and 23). On the other hand, but one of the two rats inoculated on the eighth day (No. 28 which received six cultures) developed sufficient resistance to abort the infection.

TABLE 1.

IMMUNITY TESTS MADE AFTER A SINGLE INJECTION OF

2 CULTURES					6 CULTURES				
Rat No.	Interval between Injections	Test Dose of *Tr. lewisi* Blood	Result	Remarks	Rat No.	Interval between Injections	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1......	14	0.005 c.c.	⊕	1 trypanosome on third day	18.....	14	0.005 c.c	o	
2......	14	" "	o		19.....	14	" "	o	
3......	2	0.0005 "	+	Rich infection	20.....	2	" "	+	Rich infection
4......	18	" "	o		21.....	2	0.0005 "	+	" "
5......	4	0.0001 "	o		22.....	18	" "	o	
6......	9	" "	o		23.....	4	0.0001 "	⊕	Very mild infection; 1/10 per field for three days
7......	13	" "	o		24.....	9	" "	o	
8......	16	" "	⊕	Very mild infection; 1 per field for seven days	25.....	13	" "	o	
9......	3	" "	+	Rich infection	26.....	16	" "	o	
10......	8	" "	+	" "	27.....	3	" "	+	Rich infection
11......	12	" "	o		28.....	8	" "	⊕	Very mild infection; 1 per field for six days
12......	16	" "	o		29.....	17	" "	o	
13......	10	" "	o		30.....	17	" "	o	
14......	10	" "	⊕	Very mild infection, 1 per field for nine days	31.....	17	" "	o	
15......	10	" "	o		32.....	17	" "	o	
16......	10	" "	o		33.....	17	" "	⊕	Very mild infection; 1–5 per field for 4 days
17......	10	" "	o						

In the tests tabulated above, controls were inoculated each time after the experimental animals, and developed a rich and lasting infection.

o indicates no infection, i.e., immunity is present.

+ indicates a rich infection, the same as that in the controls.

⊕ indicates a mild or abortive infection characterized by the presence of very few trypanosomes per field in fresh blood preparation.

Very mild or abortive infections may occur, as will be seen, in rats inoculated even on the 10th (No. 14), 14th (No. 1), and 17th (No. 33) day, but in these the parasites were, as a rule, very scanty and persisted for but a few days. These infections correspond to those which can be produced by injecting a large dose of trypanosomal blood into rats which have recovered from a natural infection. The breaking down of the immunity in such cases is usually indicated by a mild infection of but a few days' duration.

Altogether it will be evident that immunity is produced by the injection of living cultures of *Tr. lewisi* and that this immunity is most pronounced after the lapse of about 10 days. In the experiments discussed, the smallest dose of vaccine was represented by the growth present in two culture tubes.[1] The minimum dose of the living culture, sufficient to immunize, is probably a very small fraction of that mentioned. Thus, in one experiment, four rats were inoculated with the growth corresponding to one-fourth, one-half, one, and two culture tubes, respectively. After an interval of 12 days, these rats and two controls were inoculated with 0.0001 c.c. of trypanosomal blood. The treated rats showed no infection, whereas the controls became heavily infected.

Subcutaneous injection of cultures.—As might be expected a notable difference in immunity production is obtained according to whether the vaccine is introduced intraperitoneally or subcutaneously. When introduced by the latter route a much larger dose of the culture must be given in order to obtain protection. Only one experiment with single injections, given subcutaneously, was made and is worthy of note. As shown in Table 2, the rats which received two cultures became infected as heavily as did the controls; whereas those which received six cultures at the same time showed a mild abortive infection which persisted for one and three days, respectively. Evidently a single injection given subcutaneously is much less effective than when given intraperitoneally, for while a fraction of a culture is sufficient when administered by the latter route, a much larger dose is necessary when injected under the skin.

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[1] The tubes used for cultures were small, being but 12×150 mm. After inoculation the tubes were placed in a hot room at 25° for from seven to 12 days. The growth was then taken up in 0.85 per cent salt solution and at once injected.

It may be stated in this connection that the injection of an extract from uninoculated tubes has no immunizing action.

When several injections are given subcutaneously, the resulting immunity is much more marked, since the absorption of the antigen is necessarily greater. One experiment of this kind is given in Table 2 and shows perfect protection.

TABLE 2.

IMMUNITY TESTS AFTER SUBCUTANEOUS INJECTION OF LIVING CULTURES.

Rat No.	Received	Interval before Test Dose	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1	2 cultures	18 days	0.0001 c.c.	+	Rich infection
2	" "	" "	" "	+	" "
3	6 cultures	" "	" "	⊕	Mild infection lasting 3 days
4	" "	" "	" "	⊕	" " " 1 day
					Two controls gave a rich infection
5	5 injections in 5 days each of 2 cultures	17 days	" "	o	Perfect immunity
6	5 injections in 5 days each of 2 cultures	" "	" "	o	" "
7	5 injections in 5 days each of 2 cultures	" "	" "	o	" "
					Four controls gave a rich infection

Multiple injections.—In Table 2, Nos. 5, 6, and 7 show that multiple injections of the culture given subcutaneously impart a marked degree of immunity. A number of similar tests were made, the injections being intraperitoneal, in order to obtain hyperimmunity and to test the resistance of such rats to relatively large doses of trypanosomal blood.. The rats thus immunized, while fully protected against a minimal dose of virus, on the injection of a fairly large dose, such as 0.25 c.c., may show a mild abortive infection which disappears in from two to three days. An exactly similar "break down" of immunity frequently occurs when a large dose of infective blood is injected into rats which have spontaneously recovered from an infection with *Tr. lewisi*. These abortive infections depend largely upon the dose and to some extent upon the rat itself.

The result of these tests is given in Table 3. By way of explanation it should be stated that Nos. 1–4 received in the course of 12 days three injections each of six cultures, Nos. 5–7, in 58 days, received three injections, each of two cultures, while Nos. 8–10 in the same time were also given three doses, each of four cultures and Nos. 11–13 received similar injections, each of six cultures. Nos. 14–21 were given in the course of 67 days six injections, the first four receiving two cultures each time and the last four, six cultures.

TABLE 3.

IMMUNITY AFTER MULTIPLE INJECTIONS.

Rat No.	Received	Interval before Test Dose	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1.......	3 × 6 C 12 days	13 days	0.25 c.c.	⊕	Tr. present for two days
2.......	" " " "	" "	" "	⊕	" " " three "
3.......	" " " "	" "	" "	o	Full protection
4.......	" " " "	" "	" "	⊕	Tr. present for two days Four controls gave rich infection
5.......	3 × 2 C 58 days	5 days	0.1 c.c.	o	
6.......	" " " "	" "	" "	o	
7.......	" " " "	" "	" "	⊕	Tr. present for four days
8.......	3 × 4 C " "	" "	" "	o	
9.......	" " " "	" "	" "	o	
10.......	" " " "	" "	" "	o	
11.......	3 × 6 C " "	" "	" "	o	
12.......	" " " "	" "	" "	o	
13.......	" " " "	" "	" "	o	Two controls for this set gave rich infection
14.......	6 × 2 C 67 days	7 days	0.005 c.c.	o	This set of eight were injected two weeks later with 0.25 c.c of virulent blood. All resisted perfectly this secon injection
15.......	" " " "	" "	" "	o	
16.......	" " " "	" "	" "	o	
17.......	" " " "	" "	" "	o	
18.......	6 × 6 C " "	" "	" "	o	
19.......	" " " "	" "	" "	o	
20.......	" " " "	" "	" "	o	
21.......	" " " "	" "	" "	o	Four controls for this set gave rich infection

DURATION OF IMMUNITY.

Since a single injection of the living culture protects against a surely infective dose of *Tr. lewisi*, it was of interest to ascertain the duration of such immunity. This active immunity becomes manifest in about 10 days and once developed it probably persists until the death of the animal. Up to the present time we have not tested out the immunity for a longer period than five months, but there can be little doubt that it will be found to last for a considerably longer period.

In Table 4, which may be looked upon as a continuation of Table 1, are brought together the several tests in which the interval between the injections exceeds 18 days. The several groups were tested at different times with the usual dose of 0.0001 c.c. of trypanosomal blood. In every case controls were inoculated at the same time and invariably gave a rich infection.

TABLE 4.

Duration of Immunity Following the Single Injection of Living Cultures.

Rat No.	Received	Interval before Test Dose	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1.......	2 cultures	35 days	0.0001 c.c.	0	
2.......	" "	" "	" "	0	
3.......	6 "	42 "	" "	0	
4.......	" "	" "	" "	0	
5.......	6 "	74 "	" "	0	
6.......	" "	" "	" "	0	
7.......	2 "	78 "	" "	0	
8.......	" "	" "	" "	⊕	A single trypanosome was found on each of two days
9.......	" "	84 "	" "	0	
10.......	" "	" "	" "	0	
11.......	" "	112 "	" "	0	
12.......	6 "	119 "	" "	0	
13.......	2 "	150 "	" "	0	
14.......	6 "	159 "	" "	0	

IMMUNIZATION AGAINST NATURAL INFECTION.

The marked protection following the introduction of living cultures suggested the possibility that vaccinated rats would be equally protected against natural infection by the flea or louse. The number of parasites present in the test dose of blood employed in the previous experiments must be considerably larger than can be introduced through the agency of a biting insect. If, therefore, no other factor is involved, it should be possible to protect the rats against the natural infection by means of insects.

The white rat which we have used exclusively has never been found in this laboratory to harbor fleas and consequently we have been unable to undertake experiments with this insect. The rats, however, are often heavily infected with lice, and since, unquestionably, these as well as fleas are agents of transmission, we endeavored to protect the rats against this source of infection.

In our first experiment we tried to secure infection by the direct transfer of lice from infected rats to a set consisting of four clean and four vaccinated rats, each of the latter having previously been given an injection of six cultures. Although considerably over 100 lice were transferred to each rat, no infection followed. Later on, therefore, these rats were confined in a small space with infected rats which were heavily infested with lice but the result was no better. At the end of five months, although blood examinations were made twice weekly for over four months, no trypanosomes could be detected in the exposed rats. The failure of the controls to become infected was not due to an unrecognized infection, since one of these inoculated on the 133d day with 0.0001 c.c. of virulent blood gave a typical infection.

For the second experiment 10 rats were injected each with six cultures. Five of these (see Table 1, Nos. 29–33) were tested 17 days later and found to be immune. The remaining five, together with five clean rats for control, and five rats containing *Tr. lewisi* and heavily infested with lice, were placed in a small cage where they remained for several months. The rats were examined at least once a week for two months but no spread of the infection occurred.

A third experiment was more successful. As above, 10 rats were first injected each with two cultures and after 10 days, five of these were tested and found to be immune (see Table 1, Nos. 13–17). The remainder, together with five clean and eight lousy, infected rats, were confined in a small cage. Later five additional infected and lousy rats were added. Of the vaccinated rats four died on the 24th, 29th, 52d, and 130th day, respectively, without at any time showing trypanosomes in their blood. Of the five clean controls, two died on the 61st and 106th day without becoming infected. The remaining three became infected on the 28th, 69th, and 81st day. The fact that three out of five non-immunes and none of the vaccinated animals became infected would indicate that the living vaccine does protect against infection by the louse. Further tests, however, especially with directly transferred lice, will be necessary fully to establish this point. It seems from the work done thus far that the lice from young rats which have been inoculated with *Tr. lewisi* but a few days before are more likely to convey the infection than those from rats having an old chronic infection.

CURATIVE ACTION OF CULTURES.

The readiness with which the living culture immunizes against infection opens the possibility of using such material to abort, or in other words, cure an infection already under way. The extremely rapid and heavy infection in the case of *Tr. lewisi* makes this at the outset a very unpromising attempt; for when a relatively small dose of trypanosomal blood, as shown in Table 3, may serve to break down an existing immunity it is very probable that the enormous number of parasites in the infected animal will exert a corresponding action and hence completely overwhelm any resistance which might be produced by the vaccine. Possibly repeated inoculation of large quantities of vaccine might eventually give rise to an amount of antibodies sufficient to check the parasites present.

Before, however, undertaking to put an end to a well developed infection, it was desirable to ascertain whether it was possible to check an infection at the very beginning; in other words, to prevent it by the simultaneous injection of virus and vaccine. In one experiment of this kind, three young rats received an intraperitoneal injection of 0.0001 c.c. of trypanosomal blood. Two of these were reserved for controls while the third was given, likewise intraperitoneally, within an hour, a suspension of the growth from 50 cultures. A fourth rat also received 50 cultures and served as control. It showed a few abortive trypanosomes on the first two days but otherwise no sign of infection. Trypanosomes did not appear in the blood controls until the third day, and then in very small numbers, about one in every 10 fields of the fresh blood. In the treated rat, on the contrary, they appeared on the first day, were two per field on the second, and four per field on the third day. Some blood drawn from the tail of this rat on the second day and injected into another produced a typical infection, thus demonstrating that the trypanosomes present were not of the abortive type. The appearance of the trypanosomes in the treated rat so much earlier than in the untreated controls is an interesting fact which was corroborated in the second experiment.

Although the trypanosomes in the untreated controls appeared later than in the treated rat, yet, once present, they multiplied more rapidly and in three days were several times more numerous than in the latter. On the seventh day they approximated, roughly speaking, about 200 per field, and this rich infection was maintained during the following three weeks. In the treated rat the maximum number was about 100 per field, on the eighth to the 11th day; the parasites then decreased and disappeared on the 30th day, at which time both controls still showed a maximum infection. This experiment indicated that the injection of a sufficiently large dose of vaccine, shortly after the infective dose, modifies somewhat the course of the infection. The result, however, was not as sharp and conclusive as might be desired.

Consequently, a second experiment was made. Two rats were given an infective dose of 0.0001 c.c., as before. One of these was given 100 cultures in three injections, during the next three hours. In this treated rat, as in the one mentioned above, trypanosomes were found on the first and second days, at which time none could be found in the control. Stained preparations made at this time showed that the trypanosomes were nearly all of the large "female" type, many of which were undergoing active division as indicated by the presence of double nuclei, blepharoplasts, and even of double flagella. Minute flagellates, such as are formed by the breaking up of the eight-celled rosette, were also noted. Of special interest is the fact that though the trypanosomes appeared earlier than in the control they did not continue to increase as they did in the latter.

In the treated rat, the trypanosomes at first appeared to be stimulated to very rapid multiplication, but this was soon checked and the number was greatly decreased, so that on the sixth and seventh days, on an average, not more than one could be found in every two fields of a fresh blood preparation. After this there was a gradual rise and although the rat was examined for 26 days the number never exceeded about 75 per field. The course of the infection in this treated rat, even more so than that in the first experiment, is indicative of a marked inhibitory influence and points to the possibility of aborting an infection by repeated large doses of the vaccine.

The course of the infection in the rats of this last experiment can best be seen from the following summary. The figures here given express roughly the number of trypanosomes present, per field of the No. 7 objective, of a fresh blood preparation. Thus, 1/10 is to read as one trypanosome in 10 fields; 3 as that many per field, etc.

Inoculated	Examined											
Feb. 26	Feb. 27	Feb. 28	Feb. 29	Mar. 1	Mar. 2	Mar. 3	Mar. 4	Mar. 5	Mar. 6	Mar. 7	Mar. 8	Mar. 9
Treated rat...	1/10	3	10	10	5	½	½	5	10	10	25	50
Control rat...	0	0	1/20	15	75	100	150	200	200	200	250	Dead

PASSIVE IMMUNITY.

Since the animals treated with one or more injections of living cultures develop a very marked immunity, it was to be expected that their blood would carry antibodies, and consequently no special effort has as yet been made otherwise to demonstrate their presence. Three tests, however, of a preliminary character, were made with the blood of rats which had received several spaced injections. These tests, like the curative experiments given above, were intended to ascertain the effect of simultaneous injections of virus and hyperimmune blood. The results, it will be seen, are by no means satisfactory.

Experiment 1.—The immunized rat received, in the course of 28 days, seven injections, each of four cultures. After an interval of 153 days, 1 c.c. of blood was drawn from the heart and injected into a clean young rat. This was followed at once by an injection of 0.0001 c.c. of trypanosomal blood. A like dose of the virus was given at the same time to two controls and to the hyperimmune rat. The controls developed the typical rich infection, whereas the latter, as expected, resisted perfectly, although five months had passed since the last protective inoculation. The rat which received the dose of immune blood became infected the same as the controls. Apparently, the blood was without protective action.

Experiment 2.—The immunized rat received four injections, of six cultures each. Seventeen days after the last inoculation it was bled and two rats were injected with the blood, receiving 2.0 and 0.5 c.c., respectively. Within a minute, these two rats and two controls were each given 0.0001 c.c. of trypanosomal blood. The rat which received 2 c.c. of the immune blood had a period of incubation of five days; the other three rats showed parasites on the third day. Otherwise, there was no difference, the severity of the infection in the treated rats being the same as in the controls. Here again, the blood seemed to be without any protective action.

Experiment 3.—The immune rat in this test had been given a total of 34 cultures in six injections. It was bled after an interval of 31 days and two rats were given respectively, 3.0 and 1.0 c.c. of its blood. This injection was followed within four minutes by the usual dose of trypanosomal blood (0.0001 c.c.). The same dose of virus was given to two controls and to two young rats born of a mother which had received an injection of two cultures. The latter and the controls gave rich infections. The treated rats, while they possibly showed fewer trypanosomes on the first two days of the infection, eventually gave as rich infections as the controls.

It is evident that in these three tests passive immunity was not conferred. Had the immune blood been injected one or two days previous to the infective dose, the result might have been different. To protect against the virus, when given simultaneously, a much larger dose of blood is needed, or else the blood must come from animals which are more highly immunized.

EFFECT OF BERKEFELD FILTRATES OF PLASMOLYZED CULTURES.

Since living as well as dead plasmolyzed cultures immunize against infection with *Tr. lewisi*, it may be pertinent to inquire whether this immunity is due entirely to the solid intracellular constituents or whether soluble products are in any way concerned. In order to obtain some light on this point, a large number of cultures (120) were prepared. The growth was taken up in 0.85 per cent salt solution and transferred to collodium sacs, immersed in running distilled water, and subjected to plasmolysis for 18 hours. At the end of that time the flagellates had disintegrated, or rather shrunk to small rounded forms. The contents of the sacs were then centrifugated, at 8,000 revolutions, to remove the cell detritus, and the clear liquid was passed through a small Berkefeld filter. The filtration, aided by an aspirator, was rapid and required but a few minutes.

The clear filtrate was injected into 6 pairs of rats; the rats of each pair receiving the equivalent of 2, 4, 6, 12, 12, and 24 cultures, respectively. Twelve days later, each of these, as well as four controls, received 0.0001 c.c. of trypanosomal blood. One of the treated rats which received the equivalent of four cultures gave a mild infection which cleared up on the ninth day. All of the other treated rats developed as rich infections as did the controls and, consequently, the single exception referred to is of no significance. The only conclusion, therefore, which can be drawn from this test is that the Berkefeld filtrates of plasmolyzed cultures have no immunizing properties. It may be added, parenthetically, that the same fact has been noted by Dr. Cumming in connection with his studies (unpublished) on rabic virus. While plasmolyzed suspensions of this virus confer a marked immunity, the filtrates of such suspensions are inert. In other words, it would seem that the insoluble constituents of the cultures, as well as of the rabic virus, are essential to the production of the immunity.

SUMMARY.

Tr. lewisi after cultivation for several years on rabbit blood agar medium becomes non-infective.

Trypanosomes may appear in very small numbers in the blood of rats inoculated with such cultures but they are incapable of multiplication *in vitro* or *in vivo*.

Rats which receive one or more injections of the living culture acquire a solid immunity which becomes apparent in about 10 days.

The immunity produced by the living culture is lasting and probably persists through life.

The immunity induced by the living vaccine is probably efficacious against the natural transmission through the flea and louse.

The immunity is not due to soluble products, since plasmolyzed and filtered cultures are inert.

This successful immunization against *Tr. lewisi* by means of a living vaccine renders it probable that like results may be obtained with the more strictly pathogenic trypanosomes. It may be added that encouraging results have already been obtained with *Tr. brucei*.